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Comparison of the peripheral blood micronucleus test using flow cytometry in rat and mouse exposed to aneugens after single-dose applications

Zoryana Cammerer*, Azeddine Elhajouji, Micheline Kirsch-Volders[1] and Willi Suter

Safety Profiling and Assessment, Exploratory Development, Novartis Pharma AG, MUT-2881.5.44, CH4002 Basel, Switzerland and [1]Laboratory of Cell Genetics, Vrije Universiteit Brussel, Brussels, Belgium

Detection of clastogenic compounds in the peripheral blood micronucleus test (MNT) in rats is a well-established methodology. However, the results obtained on the induction of micronuclei by aneugens in rat peripheral blood are controversial. Our aim was a comparative evaluation of the peripheral blood flow cytometry MNT in Wistar Han rat and CD1 mouse exposed to three aneugens (vinblastine, vincristine and colchicine) after single-dose applications. In addition, the same compounds were tested in the rat bone marrow MNT. The treatment with vinblastine (0.25, 0.5, 1, mg/kg), vincristine (0.025, 0.05, 0.1 mg/kg) or colchicine (0.7, 1, 1.3 mg/kg) induced no statistically significant increase in MN-PCEs (micronucleated polychromatic erythrocytes or reticulocytes) in rat peripheral blood. In rat bone marrow, a clear statistically significant increase in MN-PCE was found with vincristine and vinblastine. However, colchicine showed a clear increase in MN-PCE frequency without reaching statistically significant level only at 1 mg/kg. The positive effect in the bone marrow MNT shows that the target organ was exposed to the appropriate concentration levels of the respective aneugens. In mouse, the peripheral blood flow cytometry analysis after the treatment with vinblastine, vincristine and colchicine showed clear statistically significant increase in MN-PCE with all three compounds. The experiments with splenectomized rats treated with vincristine and colchicine were performed and statistically significant increases in MN-PCE were found with 0.05, 0.1, 0.15 mg/kg of vincristine and 0.7 and 1 mg/kg of colchicine. Our results demonstrate that micronucleated cells induced by aneugens are removed from rat peripheral blood by the spleen due to the large size of micronuclei. Based on our data, it is concluded that the flow cytometry peripheral blood MNT after single-dose applications is an appropriate test system for evaluating the genotoxic effects of aneugens in mice. However, in rats peripheral blood MNT aneugen detection might require multiple-dose applications to overwhelm the spleen effect.

Introduction

The bone marrow micronucleus test (MNT) is a standard assay used in genetic toxicology (1–3). Using peripheral blood instead of bone marrow has several advantages including easy sample preparation, ability to obtain repeat samples from the same animal and ability to obtain samples from chronic toxicology studies. The mouse is the rodent species most frequently used for the MNT but rat is the standard species used in toxicology and pharmacokinetics. However, there was a limitation in using peripheral blood erythrocytes since the spleen selectively removes micronucleated (MN) erythrocytes from circulation of many species (4,5). The removal of MN erythrocytes was proven for the rats (6) and humans (7) but MN erythrocytes persist in mouse peripheral blood (8,9). Studies with the clastogens mitomycin C and cyclophosphamide showed that rat peripheral blood reticulocytes can be used as reporter cells for the detection of micronucleus induction in bone marrow (10,11). However, aneugens and clastogens have different mechanisms of action. Aneugens investigated in this study induce aneuploidy by interacting with tubulin and inhibiting the polymerization process necessary for mitotic spindle formation. This process may lead to the loss of whole chromosomes. However, clastogens may cause chromosome strand breaks and result in the loss of chromosomal fragments. Therefore, it is expected that the micronuclei induced by aneugens are larger in size (i.e. containing whole chromosome) than micronuclei induced by clastogens (i.e. containing chromosome fragments). To investigate the size-based differential spleen elimination of micronuclei induced by aneugens in rat peripheral blood, a comparative evaluation of the peripheral blood flow cytometry MNT in rat and mouse exposed to aneugens was performed. The mouse is used as a reference rodent model since the elimination of the MN reticulocytes by the spleen is not occurring.

Materials and methods

Animals

Male Wistar Han rats and CD1 mice 7 weeks old were purchased from Charles River Laboratory (Sulzfeld, Germany). Splenectomized rats 8 weeks old were purchased from Charles River Laboratory (L'Arbresle, France). The spleen was removed when the animals were 6 weeks old. The animals were acclimatized for 1 week before the start of the experiments. Food and water were available *ad libitum*. Twelve animals were used for each experiment, three animals for each dose group including negative controls.

Chemicals and reagents

Vincristine sulphate (CAS number: 2068-78-2), vinblastine sulphate (CAS number: 43-67-9) and colchicine (CAS number: 64-86-8) were purchased from Sigma–Aldrich (Buchs SG, Switzerland). Vehicle NaCl 0.9% was purchased from B. Braun Medical AG (Emmenbrücke, Switzerland). MicroFlow Rat and Mouse Micronucleus Analysis Kits were purchased from Litron (NY, USA). The kits contained solution A, methanol; solution B, heparin; solution C, washing buffer [Hanks' balanced salt solution (HBSS)]; solution D, RNase; solution E, anti-CD71 antibody; solution G, anti-platelet antibody (phycoerythrin); solution F, propidium iodide (PI); malaria biostandard blood samples and positive and negative control samples.

Treatment protocols

In all experiments, single treatments were performed. The compounds were dissolved in NaCl 0.9% and administered intraperitoneally (i.p). The dose range selection for the respective compounds was based on preliminary toxicity

*To whom correspondence should be addressed. Tel: +41 61 3241459; Fax: +41 61 3241274; Email: zoryana.cammerer@novartis.com

assessment. The rat peripheral blood sampling was performed at 24, 30, 48 and 72 h for vinblastine and vincristine experiments. In colchicine experiment, blood sampling was performed at 24, 30 and 48 h due to animal sacrifice for parallel bone marrow collection but at 24, 30, 48 and 72 h for splenectomized rat experiment. For the experiments, rat bone marrow and mouse peripheral blood samples were collected at 48 h.

Blood sample preparation

Peripheral blood samples were obtained for flow cytometric analysis using the MicroFlow Rat Micronucleus Analysis Kit (Litron) by following the three-colour staining protocol. Approximately, 120 µl of blood was collected from the tail vein of each animal into a tube containing 350 µl of heparin. Blood samples were maintained at room temperature for no more than 3 h before fixation. Samples were fixed in ultracold (-80°C) methanol. For the fixation, 180 µl of blood was added to a polypropylene centrifuge tube filled with 2 ml ultracold methanol, mixed and stored at -80°C until analysis. On the day of analysis, the fixed blood samples were removed from the -80°C freezer and tapped gently several times and 12 ml of an ice-cold solution C was added. The cells were isolated by centrifugation at approximately $600\times$ *g* for 5 min. Cell pellets were stored at +4°C. Samples were shortly re-suspended by vortexing; 20 µl aliquots of fixed blood cells were added to tubes containing 80 µl of the staining solution (10 µl of the solution D and E, 5 µl of the solution G per millilitre of the solution C). The samples were kept for 30 min at 4°C and for 30 min at room temperature to ensure the complete degradation of the RNA. The samples were stored at 4°C until analysis. Directly before analysis, cells were diluted with 2 ml cold PI staining solution.

Flow cytometry analysis

Flow cytometry micronucleus test is based on an immunochemical reagent (anti-CD71–fluorescein isothiocyanate) which differentially labels polychromatic and normochromatic erythrocytes, PI staining to detect micronucleus in poly- and normochromatic erythrocytes and PE antibody used to exclude platelet aggregates. Young PCEs or reticulocytes still have CD71 antigen on their surface; however, with the maturation process the amount of the CD71 antigen rapidly decreases. Staining of the erythrocytes with anti-CD71 antibodies allows the selection of the young PCEs containing CD71 antigen for further analysis by flow cytometry.

All flow cytometric analyses were carried out with a FACS Calibur flow cytometer tuned to 488-nm excitation. A total of 20 000 MN-PCEs were analysed per sample. Anti-CD71, anti-platelet–PE and PI fluorescence signals were detected in the FL1, FL2 and FL3 channels, respectively. Erythrocytes infected with *Plasmodium berghei*, biological standard (purchased from Litron), were used to model micronucleus-containing cells and to set up and calibrate the instrument (12,13). The parasite-infected blood was stained in parallel with the test samples, so that adjustments to flow cytometer settings could be made. While analyzing a malaria-infected blood sample, photomultiplier tube voltages and laser power were adjusted to maximize resolution between cells with and without malaria parasites, and also between cells with single and multiple parasites.

Bone marrow sample preparation

For each rat, a disposable plastic tube (5 ml) is filled with 2 ml filtered 25 mM ethylenediaminetetraacetic acid (EDTA)/fetal calf serum (FCS) mix, and a 2-ml plastic syringe with a disposable needle was prepared. Two glass slides coated with poly-L-lysine were used for each animal. The animals were killed by CO_2 asphyxiation. Immediately after death, one femur was dissected and the content was drawn into a centrifuge tube with 2 ml of 25 mM EDTA/FCS mix. Samples are prepared according to the method described in the literature (14,15).

Removal of nucleated cells from bone marrow

Cellulose columns were prepared as follows: equal parts by weight of microcrystalline cellulose, Sigmacell type 50. For each animal, 400–450 mg of the cellulose mixture was filled into a disposable plastic syringe without plunger. The syringe was sealed by placing an 8-µm disk filter membrane between the syringe tip and the attached needle. As soon as the cell suspension has entered the cellulose matrix, 1.5 ml HBSS (without phenol red) was applied to the column surface.

Cell separation

Eluates were loaded directly from the columns onto 35% Percoll solutions. These solutions were then centrifuged for 10 min at 2200 r.p.m. The pellet was taken up, washed with 10 ml HBSS (5 min, 2000 r.p.m.) and re-suspended in FCS with 50 mM EDTA. The cell number was determined by a Sysmex Cell Counter and diluted to approximately 5–8 $\times$ 10^6 cells/ml.

Cytocentrifugation to flatten cells

Cytocentrifugation was carried out in a Shandon Cytospin. An aliquot of the cell suspension (50–100 µl) was loaded into the chamber. The cells were then pelleted onto slides coated with poly-L-lysine at 1400 r.p.m., high acceleration, for 7 min. Slides were left to dry at room temperature for at least 12 h before staining and then stained by May-Gruenwald/Giemsa solution.

Bone marrow slide analysis

The slides were analysed automatically using an image analysis system (ROBIAS image analyser). Following the scoring process, the MN-PCEs identified by the system were examined for possible artefacts. For each rat, two slides per animal were analysed. A total of 2000 PCEs per slide are analysed for micronuclei, i.e. 4000 PCEs per animal.

Data analysis

Mean and standard deviation for PCE% and MN-PCE% were calculated for each treatment group. The results of the MNT were evaluated according to the following criteria: a compound was classified as genotoxic in the rat MNT if it induced a statistically significant increase in micronucleus frequency above the control level. The statistical evaluation was performed with the SAS software package (SAS version 8.02). Levene's test for homogeneity of variances was performed on absolute deviations (16). If variances were not significantly heterogeneous (17), then a one-way analysis of variance (ANOVA) was performed (18). If the ANOVA detected significant differences between groups, a multiple comparison with the controls was performed with the Dunnett test (19). If the variances were significantly heterogeneous, a non-parametric analysis was performed using the Kruskal–Wallis test (20). A multiple comparison with the control group was done using the Dunn test (21). The significance level used was $P \leq 0.05$.

Results

Rat peripheral blood experiments

The aneugens, vinblastine, vincristine and colchicine, were applied i.p. as a single treatment with doses 0.25, 0.5 and 1 mg/kg of vinblastine; 0.025, 0.05 and 0.1 mg/kg of vincristine and 0.7, 1 and 1.3 mg/kg of colchicine. No statistically significant increases in micronucleus frequency were found in rat peripheral blood for all three compounds at any time point (Figures 1, 2 and 3). However, the dose of 1.3 mg/kg of colchicine was not analysed at 48 h due to the strong symptoms of toxicity and animal mortality.

Rat bone marrow experiments

Bone marrow experiments were performed with vinblastine, vincristine and colchicine at the same doses as the peripheral blood experiments. Bone marrow collection was performed at 48 h after the single treatment. Vinblastine at 0.5 and 1 mg/kg (Figure 4) and vincristine at 0.1 mg/kg (Figure 5) induced

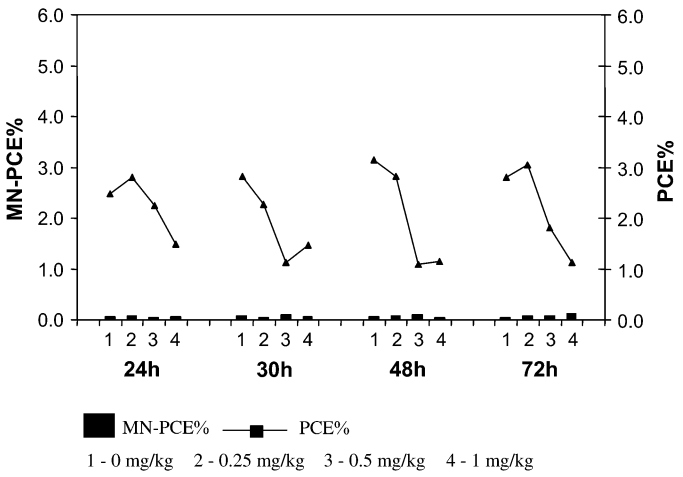

Fig. 1. Frequencies of PCE% and MN-PCE% in rat peripheral blood after the single treatment with vinblastine 0, 0.25, 0.5 and 1 mg/kg.

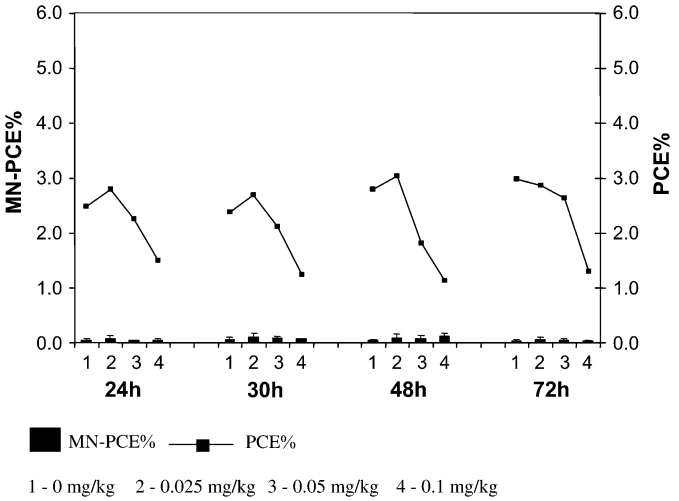

Fig. 2. Frequencies of PCE% and MN-PCE% in rat peripheral blood after the single treatment with vincristine 0, 0.025, 0.05 and 0.1 mg/kg.

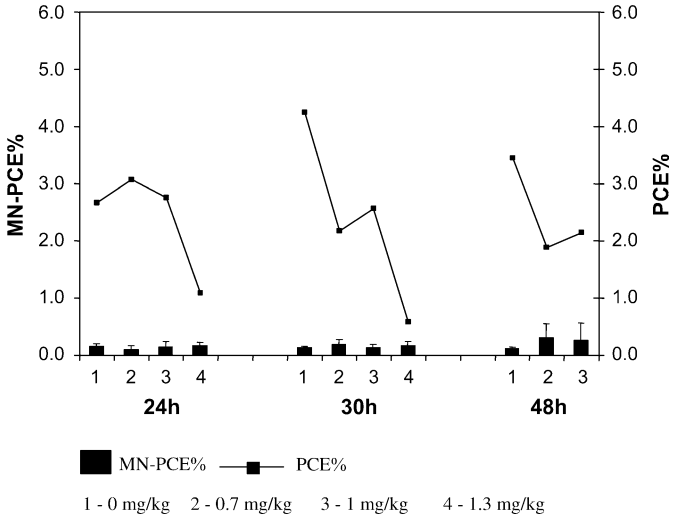

Fig. 3. Frequencies of PCE% and MN-PCE% in rat peripheral blood after the single treatment with colchicine 0, 0.7, 1 and 1.3 mg/kg. The dose of 1.3 mg/kg of colchicine was not analysed at 48 h due to the strong symptoms of toxicity and animal mortality.

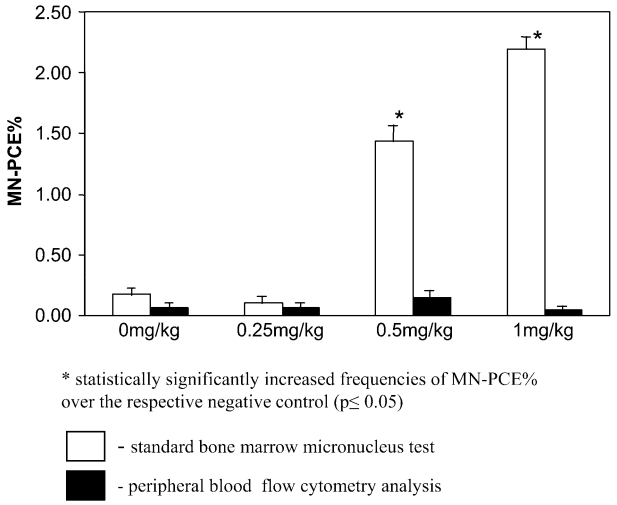

Fig. 4. Comparison of the rat peripheral blood and bone marrow analysis after treatment with vinblastine. Blood and bone marrow collection was performed at 48 h after the single treatment. *Statistically significantly increased frequencies of MN-PCE% over the respective negative control ($P \leq 0.05$)

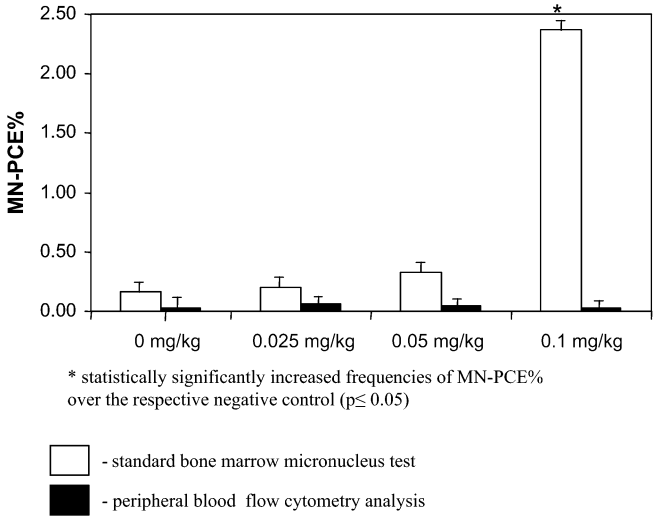

Fig. 5. Comparison of rat peripheral blood and bone marrow analysis after treatment with vincristine. Blood and bone marrow collection was performed at 48 h after the single treatment. *Statistically significantly increased frequencies of MN-PCE% over the respective negative control ($P \leq 0.05$)

a clear statistically significant increase in MN-PCE in the bone marrow. The marginally elevated average MN-PCE after the treatment with colchicine at 1 mg/kg was not statistically significant and would require more animals per group to reach statistical significance given the large inter-individual variability that was observed. The dose of 1.3 mg/kg of colchicine was not analyzed due to the strong symptoms of toxicity and animal mortality (Figure 6).

Mouse peripheral blood experiments

After a single i.p. application of vinblastine (0.25, 0.5, 1 mg/kg), vincristine (0.025, 0.05, 0.1 mg/kg) and colchicine (0.25, 0.5, 1 mg/kg), blood collection was performed at 48 h after the treatment. The two highest doses of vinblastine (0.5, 1 mg/kg) and vincristine (0.05, 0.1 mg/kg) and 1 mg/kg of colchicine showed a clear statistically significant increase in MN-PCE% (Table 1).

Splenectomized rat experiments

Splenectomized Wistar Han rats were treated via single i.p. injection with 0.05, 0.1 and 0.15 mg/kg of vincristine and 0.7, 1 and 1.3 mg/kg of colchicine. Blood collection was done at 24, 30, 48 and 72 h. All three doses of vincristine (Figure 7) and 0.7, 1 mg/kg of colchicine (Figure 8) showed statistically significant increases in MN reticulocytes at 48 h after treatment. The dose of 1.3 mg/kg of colchicine was not analysed at 48 and 72 h due to the strong symptoms of toxicity and animal mortality.

Discussion

One of the main advantages of the MNT is the ability to evaluate the clastogenic and aneugenic potential of compounds using the same end point *in vitro* and *in vivo*. Early studies showed that the size of micronuclei can be used as a possible

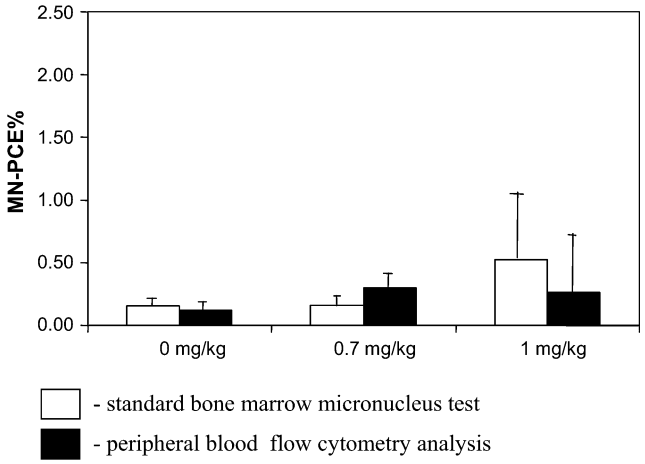

Fig. 6. Comparison of rat peripheral blood and bone marrow analysis after treatment with colchicine. Blood and bone marrow collection was performed at 48 h after the single treatment. The dose of 1.3 mg/kg of colchicine was not analysed due to the strong symptoms of toxicity and animal mortality.

Table I. Frequencies of PCE% and MN-PCE% in mouse peripheral blood after the single treatment with vinblastine, vincristine and colchicine

Compound, dose	MN-PCE%	PCE%
Negative control NaCl, 0.9%	0.17 ± 0.02	1.91 ± 0.02
Vinblastine, 0.25 mg/kg	0.34 ± 0.18	1.58 ± 0.02
Vinblastine, 0.5 mg/kg	1.45 ± 0.45*	1.01 ± 0.02
Vinblastine, 1 mg/kg	1.74 ± 0.36*	0.47 ± 0.04
Negative control NaCl, 0.9%	0.37 ± 0.10	1.29 ± 0.01
Vincristine, 0.025 mg/kg	0.58 ± 0.34	1.68 ± 0.04
Vincristine, 0.05 mg/kg	1.01 ± 0.45*	1.61 ± 0.01
Vincristine, 0.1 mg/kg	2.74 ± 0.50*	0.38 ± 0.01
Negative control NaCl, 0.9%	0.18 ± 0.04	2.36 ± 0.02
Colchicine, 0.025 mg/kg	0.14 ± 0.02	1.67 ± 0.01
Colchicine, 0.05 mg/kg	0.30 ± 0.09	1.89 ± 0.04
Colchicine, 0.1 mg/kg	1.22 ± 0.49*	1.01 ± 0.05

*Statistically significantly increased frequencies of MN-PCE% over the respective negative control ($P \leq 0.05$).

parameter to distinguish clastogens (small micronuclei) from aneugens (large micronuclei) (22–24). However, the elimination of the MN reticulocytes by the spleen can be a limiting factor in the rat peripheral blood MNT for the detection of aneugens (6), and the size of the micronucleus can play an important role in the elimination process. Such an effect by the spleen was not observed in the mouse (8,9).

In our study, we performed a comparative evaluation of the peripheral blood flow cytometry MNT in rats and mice exposed to single dose of the following aneugens: vinblastine, vincristine and colchicine. Vinca alkaloids vinblastine and vincristine are important at cancer treatment. Colchicine is mainly indicated for the treatment and prophylaxis of gout and familial Mediterranean fever.

In rat peripheral blood, no induction of micronuclei was found after single treatment with vincristine, vinblastine and colchicine. In the same time, the rat bone marrow MNT showed a clear positive effect indicating that the target organ for haemopoiesis, i.e. bone marrow, was exposed to the respective compounds. A literature review showed that vincristine, vinblastine and colchicine were previously evaluated in different experimental set-ups and showed contradictory

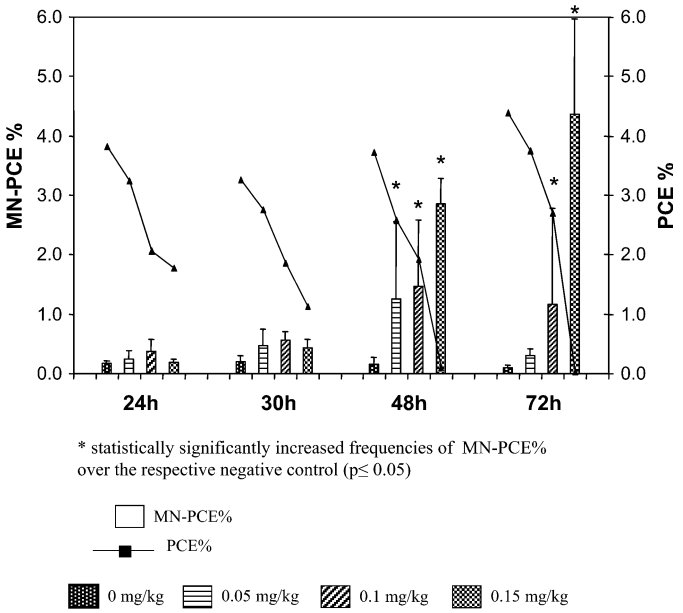

Fig. 7. Frequencies of PCE% and MN-PCE% in peripheral blood of splenectomized rats after the single treatment with vincristine 0, 0.05, 0.1 and 0.15 mg/kg. *Statistically significantly increased frequencies of MN-PCE% over the respective negative control ($P \leq 0.05$).

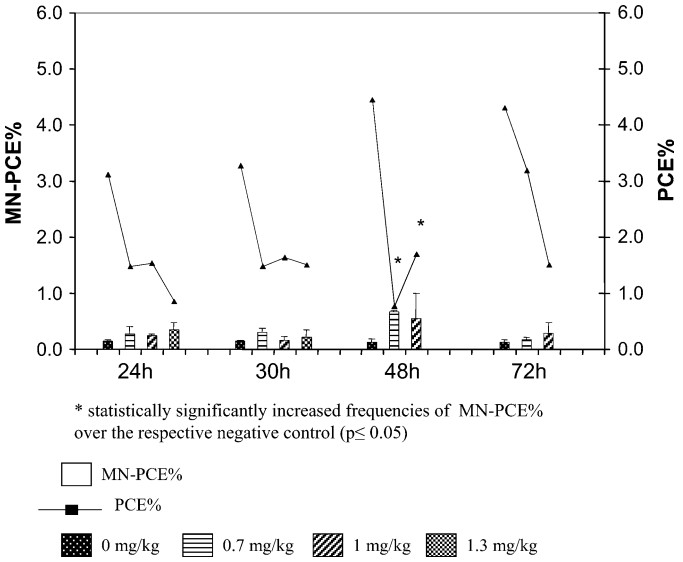

Fig. 8. Frequencies of PCE% and MN-PCE% in peripheral blood of splenectomized rats after the single treatment with colchicine 0, 0.7, 1 and 1.3 mg/kg. The dose of 1.3 mg/kg of colchicine was not analysed at 48 and 72 h due to the strong symptoms of toxicity and animal mortality. *Statistically significantly increased frequencies of MN-PCE% over the respective negative control ($P \leq 0.05$).

results. A study by Wakata et al. (25) showed inconclusive results in the peripheral blood of Sprague–Dawley (SD) and Fisher 344 rats after the treatment with colchicine; however, positive results were obtained in rat bone marrow. Acridine orange staining was used in this study to identify micronuclei. In a study by Hynes et al. (26) using flow cytometry, colchicine was found negative in peripheral blood of Wistar Han rats up to 6 mg/kg after single oral or i.p. treatment. However, in peripheral blood of CD1 mice, positive results were shown at 1 and 2 mg/kg. "Study by Asano et al. (27) with CD-1 and MS/Ae

mice showed increase in micronucleus frequency in mouse bone marrow at 24 h after i.p. administration of 1 mg/kg colchicine in both strains." Vinblastine was found positive in F1 mice in peripheral blood MNT by Grawe et al. (28). Studies with vincristine using the three different moue strains CD1, MS/Ae or ICR after i.p. administration showed an increase in micronucleus frequency in bone marrow at 0.125 mg/kg in CD1 mice, 0.25 mg/kg in MS/Ae mice (29) or 0.05 mg/kg in ICR mice (30). It is clear from our data and the data published in the literature that it is possible to detect aneugens in mouse peripheral blood and also in mouse and rat bone marrow MNTs. However, the detection of aneugens in rat peripheral blood is still questionable. The experiments with splenectomized rats bring further evidence to the micronucleus elimination process that occurs in the rat spleen. Indeed a comparative evaluation of aneugen-induced MN-PCE frequencies in the peripheral blood of mice, normal rats and splenectomized rats indicate that the spleen plays an important role in rats but not in mice. However, in mouse and rat peripheral blood, statistically significant increases in MN-PCE frequencies were observed after exposure to clastogenic compounds (31,32). This difference between clastogens and aneugens suggests that the removal of the MN cells by the spleen from blood circulation of rats is probably due to the large size of micronuclei. In two studies published by Abramsson-Zetterberg et al. (33) and Torous et al. (34), significant increases in micronucleus frequencies were found in rat peripheral blood after vincristine administration. In the study by Abramsson-Zetterberg et al. (33) the flow cytometry analysis of MN-PCE (using Hoechst 33342 and Thiazole Orange staining) in peripheral blood, bone marrow and spleen of SD rats showed statistically significant increase after single treatment with 0.05 and 0.1 mg/kg of vincristine. In the study by Torous et al. (34), the flow cytometry analysis of MN-PCE (using anti-CD71 antibody and PI staining) in peripheral blood of SD rats showed statistically significant increase after two treatments with 0.05 and 0.1 mg/kg of vincristine. However, in these studies different rat strains (SD rats), detection methods (Hoechst 33342 and Thiazole Orange) or different number of treatments were used.

Based on our data, it was not possible to detect the expected positive aneugenic effect with flow cytometry peripheral blood MNT using Wistar Han rats and following single treatment protocol. However, multiple treatment protocols have been shown to be suitable for aneugen detection in rat peripheral blood (32,35,36). This is an important issue that shows that the rat peripheral blood MNT can be integrated into chronic toxicology studies that involve repeat dosing protocols. However, the mouse peripheral blood MNT with flow cytometry using single treatment protocol is an efficient test system for the detection of aneugens. The aneugen detection in rat peripheral blood may be limited by a number of reasons, one of which is the elimination of the large MN reticulocytes by the spleen. Our results from the mouse peripheral blood and spleenectomized rats support this spleen effect. Moreover, it was suggested by MacGregor et al. (35) that the flow cytometry analysis of the youngest fraction of reticulocytes does not reduce the eliminating effect of the spleen. The detection of aneugens *in vivo* can be compromised by additional parameters characteristic of aneugens that should be taken into account. Such parameters may include a very narrow dose range between genotoxic and cytotoxic effects at the target organ that may require multiple-dose applications at lower doses to assure continuous exposure to overwhelm the spleen effect. A lack of sufficiently optimized time frame between sampling times related to cell cycle delay and therefore a delay in the release in the blood circulation of MN reticulocytes is another important factor to take into account. In addition, we assume that more animals per group might be needed to avoid the large inter-individual variability observed after treatment with aneugens, e.g. colchicine.

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